A Quantitative Stable-Isotope LC−MS Method for the Determination of Folic Acid in Fortified Foods

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A stable-isotope liquid chromatography−mass spectrometry (LC−MS) assay was developed for the quantitative determination of folic acid in fortified foods. Folic acid was extracted from food samples into a phosphate buffer, purified on a C-18 Sep-Pak cartridge, and analyzed by LC−MS in the negative ion mode using electrospray ionization. The analyte was quantified using $^{13}C_5$-folic acid as an internal standard. The coefficient of variation for the precision of the method was 5.6% based on the analysis of four sample replicates. The accuracy of the method was assessed using a standard method of addition of folic acid to a shredded whole-wheat cereal. The quantitative determination of folic acid in this matrix was linear over 1 order of magnitude having a concentration range of 2.4 to 24 μg/g of food (or 0.05 to 0.5 μg of analyte injected into the LC−MS). The overall quantitative efficiency of the method was evaluated using a standard reference material (infant formula SRM 1846). The method was applied to the determination of folic acid in several test samples (fortified breakfast cereals), and the values were in accord with the manufacturer's claim. This method advances a LC−MS technique for the determination of folic acid in fortified foods based on stable-isotope dilution methodology. The specificity of the technique and quantitative accuracy of the method in various food substrates suggests that the method may be adapted for routine analysis in other fortified foods.

Keywords: *Folic acid; mass spectrometry; fortified foods; isotope dilution; electrospray ionization*

INTRODUCTION

Folates belong to a group of heterocyclic compounds based on a 4-[(pteridin-6-ylmethyl) amino] benzoic acid structure (Figure 1). These vitamin cofactors, biosynthesized in plant tissue, are essential for the synthesis of purines, pyrimidines, and DNA and in cell replication in animals and humans. As such, they are required in the human diet (the current adult Daily Recommended Intake in the United States is 400 μg, *1*). An inadequate dietary sufficiency of folates for pregnant women has become an important public health concern. In an effort to reduce the incident of neurological birth defects (neural tube defects), the Food and Drug Administration (FDA) mandated that beginning January 1998 grain products manufactured in the United States be fortified with folic acid at a concentration of 140 μg/100 g of grain (*1*). Manufacturers subsequently initiated folic acid fortification programs in 1998 to comply with the mandate (flour, cereals, macaroni and pasta, and corn meal products are examples of some fortified foods). The proposed goal of the program was to reach compliance with the mandate by January 1, 1999.

Although some foods contain sufficient amounts of the naturally occurring folates (e.g., citrus, dark-green leafy vegetables, and whole-grain products) to furnish the nutritional requirement (*1*), most Americans consume an appreciable quantity of the vitamin in the form of folic acid from fortified food products. Unexpectedly, and as a result of the FDA mandate strengthening the fortification program, a possible public health concern might develop in another sector of society. There is some evidence that an over intake of folic acid can engender a health risk in the elderly (*2*−*3*). It has been suggested that over-fortification of foods with folic acid could mask a vitamin B-12 deficiency that may predispose older adults to additional neuropathies (*3*).

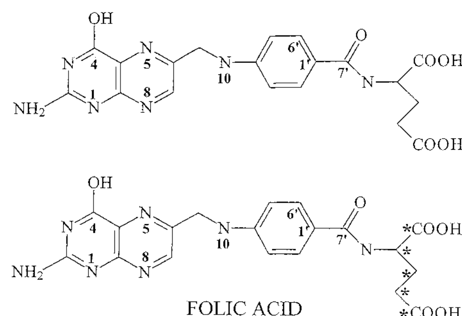

Figure 1. The structure of folic acid and $^{13}C_5$ folic acid. The vitamin consists of a pterine ring conjugated to *p*-aminobenzoic acid that is coupled to glutamic acid.

Folates belong to a class of natural products distinguished by similar chemical structures (a pterine ring conjugated to the amine of *p*-amino benzoic acid which forms an amide bond with glutamic acid, see Figure 1). The structural and chemical diversity of the naturally occurring folates prompted the development of several analytical procedures for their analyses. The assays encompass a fairly wide range of analytical procedures. A microbiological assay (*L. casiei*) has been routinely used to determine the total folate concentration in foods and biologics. The assay, based on the light-scattering

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dispersion of the flocculent growth of the organism in a folate-enriched media, is insensitive to the various chemical forms of the vitamin (*4*). Highly specific HLPC separation techniques with UV and/or fluorescent detection have been developed to detect the different vitamer forms of the folacins (*5*–*7*). A stable-isotope gas chromatography–mass spectrometry (GC–MS) procedure was developed for the determination of total folates in biological samples, whereby the folacins are chemically hydrolyzed to *p*-aminobenzoic acid, derivatized, and analyzed by GC–MS (*8*). Recently, there have been a small number of reports describing the application of LC–MS to folate analysis (*9*–*11*). Utilizing these analytical approaches folates are ionized with either atmospheric pressure chemical ionization (APCI) (*10*) or electrospray ionization (ESI) (*9*, *11*). To our knowledge, there is no current LC–MS assay for quantifying folates using a labeled analogue as an internal standard.

There is a need to develop a specific and quantitative assay for the determination of folic acid in fortified foods for the purpose of monitoring the concentration of the fortificant in the food supply, assessing compliance with the government mandate, and collecting reliable nutrient data for public health investigations. Currently, there is no definitive assay, which utilizes mass spectrometry for determining folic acid in fortified foods. The current "gold standard" method is a microbiological assay, which does not distinguish the different forms of folates and therefore is not a definitive assay for folic acid. Spectroscopic determinations alone are not definitive and therefore require alternate analyses to confirm the identity of the analyte.

We have developed a specific and selective method for the analysis of folic acid in fortified foods utilizing high performance liquid chromatography–mass spectrometry (LC–MS) with electrospray ionization. A $^{13}C_5$-isotope of folic acid (labeled on the 1, 2, 3, 4, and 5 carbon positions of glutamic acid) was used as an internal standard to quantify folic acid in foods. The accuracy of the method was evaluated using standard method of addition (SMOD) of folic acid to a whole-wheat cereal, and the method was also tested against a standard reference material (infant formula 1846, National Institute of Standards and Technology Gaithersburg, MD). A tandem mass spectrometric procedure was used to confirm the identity of folic acid in fortified foods, and the method was applied to the routine determination of folic acid in some breakfast cereals.

MATERIALS AND METHODS

Chemicals and Reagents. Monoglutamyl folic acid was purchased from Sigma (St. Louis, MO) and used without further purification. $^{13}C_5$-glutamyl-folic acid was obtained from Eprova AG (Schaffhausen, CH). The ^{13}C atoms occupied each of the carbon positions on the glutamic acid portion of the molecule. Standards were heated to 60 °C for 3 h under vacuum to remove trace amounts of water. The folic acid standards were dissolved in a 0.1 N solution of sodium hydroxide with 0.1% mercaptoethanol and stored at 4 °C. All solvents used were HPLC-grade and were obtained from commercial suppliers and used without further purification.

Sample Preparation. *Sample Preparation of Wheat, Corn, and Oat Cereals.* Dry 5-g quantities of crushed cereal were homogenized using a Sorval Omni-Mixer (Newtown, CT) containing 100 mL of 0.03 mM dibasic potassium phosphate buffer containing 0.1% each trifluoroacetic acid (TFA) and mercaptoethanol (ME) for 3 min at room temperature (at maximum velocity). Prior to homogenization, the $^{13}C_5$-labeled folic acid internal standard in 0.1 N sodium hydroxide was added to the suspension of the sample in an amount commensurate with the concentration of the analyte. Samples were purged with nitrogen and placed in a water bath that was heated to 100 °C for an hour. Following homogenization, a 10-mL aliquot of the supernatant was transferred to a 15-mL conical polypropylene centrifuge tube to which 1.5 mL of ethanol had been added. The tube was shaken on a vortex for one minute and allowed to stand at 4 °C overnight. The sample was then centrifuged at 2700*g* for 20 min at room temperature, and a 4-mL aliquot of the supernatant was pipetted for extraction using a solid-phase cartridge.

Extraction and Preparation of the Standard Reference Material (SRM) 1846. (Powdered Infant Formula) SRM 1846 was obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). The SRM 1846 was analyzed in the same manner as the cereal products with the exception that after heating and cooling to room temperature, a 4-mL aliquot of the colloidal suspension was subjected directly to solid-phase extraction without addition of ethanol.

Determination of the Effect of Heat Treatment on Folic Acid Stability. To determine the effect of heat treatment step on the stability of folic acid, several trials were carried out whereby two solutions of phosphate buffer (3 mL each) were amended with 1.3 μg of folic acid each. To one solution, $^{13}C_5$-folic acid (1.0 μg) was added and allowed to stand at room temperature for 1 h. The second solution was heated in the water bath at 100 °C for an hour prior to the addition of the labeled folic acid. Both aliquots were then subjected to solid-phase extraction as described below and analyzed by LC–MS/ESI for folic acid.

Standard Method of Addition of Folic Acid to Nonfortified Shredded-Wheat Cereal. A standard method of addition experiment was performed on a nonfortified shredded-wheat cereal. Seven aliquots (10 mL each) of a 5 g sample of shredded-wheat (homogenized in 100 mL of phosphate buffer) were amended with folic acid (ranging from 0.62 to 5.93 μg/0.5 g). Equal amounts (2.6 μg) of $^{13}C_5$ labeled-folic acid were added to each aliquot. Three additional samples in phosphate buffer alone (without the wheat cereal) were prepared using 2.0, 4.0, and 6.0 μg of folic acid together with 2.6 μg of the internal standard. These additional samples were used to determine whether the sample matrix had any effect on altering the $^{13}C_5$:^{12}C isotope ratio of folic acid and its internal standard. All samples were processed as previously described for the cereal extractions.

Analysis of Serial Dilutions of SRM 1846. To determine quantitative accuracy of the method over a range of concentrations, a 5 g sample of SRM 1846 in 100 mL phosphate buffer was divided into five aliquots and pipetted (0.5, 2.0, 4.0, 6.0, and 8.0 mL) into separate 15-mL conical tubes. The final volume of each tube was adjusted to 8 mL with the phosphate buffer and 0.39 μg of $^{13}C_5$-folic acid was added to each tube. The samples were processed as described previously for infant formula and analyzed by LC–MS.

Solid-Phase Extraction. A 4-mL aliquot of the supernatant of each extracted sample was diluted with 4 mL of 0.03 mM phosphate buffer and loaded onto a Waters (Milford, MA) C-18 Sep-Pak (100 mg) cartridge that had been previously sequentially washed with methanol (3 mL) and phosphate buffer (4 mL). After the sample was loaded onto the Sep-Pak column, the column was washed with the phosphate buffer (3 mL) and twice with water (3 mL) to remove traces of salts. The analyte was eluted with a 2 mL solution of ammonium acetate and methanol [10 mM ammonium acetate (pH 10)/methanol 78:22] into a conical centrifuge tube (5 mL) and then quantitatively transferred to a 50-mL round-bottom flask to which ethanol (10 mL) had been added. The solution was evaporated to near dryness under vacuum at 40 °C. The residue was transferred to a 5-mL centrifuge tube with ethanol and reduced to dryness under a nitrogen stream at 40 °C. To the residue was added 100 μL of the HPLC mobile phase (ACN/H_20/MEOH 26:60:14 + 0.1% formic acid and 0.1% ME). The sample was shaken on a vortex and centrifuged briefly to remove the precipitate. The supernatant was carefully transferred to an HPLC vial fitted with a 200 μL insert.

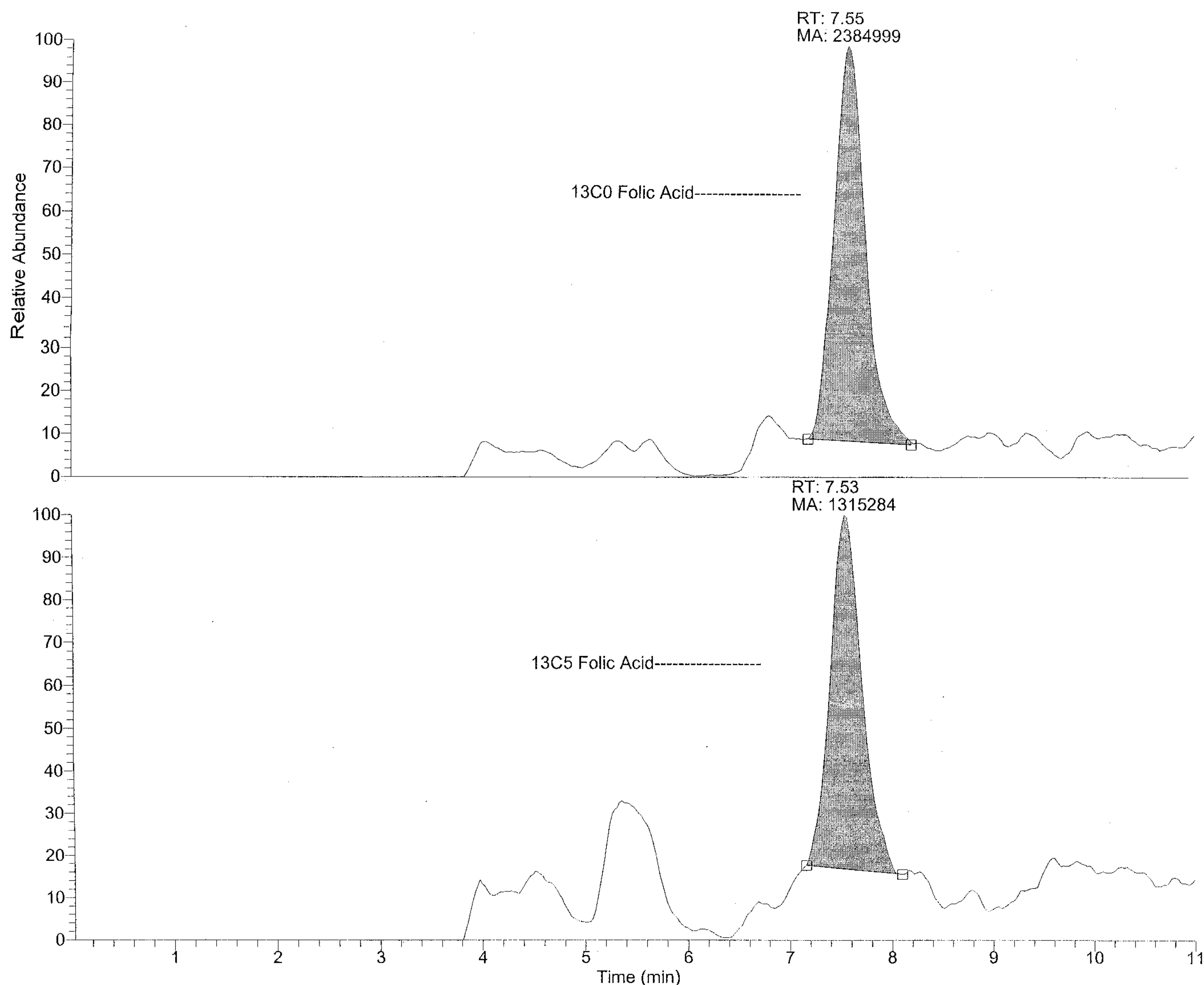

Figure 2. Selected ion chromatograms for the molecular anions for folic acid at *m/z* 440.2 and $^{13}C_5$-folic acid at *m/z* 445.2. The chromatograms are of the analysis of a corn-based breakfast cereal fortified with folic acid. The two folate compounds coelute at 7.6 min from a C-18 HPLC column using ACN/$H_2$0/MEOH 26:60:14 + 0.1% formic acid and 0.1% ME mobile phase with a rate of 0.2 mL/min.

LC/ESI-MS Apparatus and Conditions. Samples (20 μL) were injected onto a 150 × 4.6 mm Luna (Phenomenex, Torrance, CA) C-18 HPLC column (5 μ) using a binary pumped Hewlett-Packard 1100 HPLC (Palo Alto, CA) that was interfaced to an ion trap mass spectrometer (Finnigan LCQ mass spectrometer, San Jose, CA) equipped with an ESI source. The mobile phase flow was 0.2 mL min^{-1} operated at 25 °C. The mobile phase was programmed after 10 min from 14% methanol to 100% at 15 min returning to the initial mobile phase concentration at 20 min and then equilibrated for 5 min. The column effluent was diverted to the mass spectrometer 4 min after injection and analyzed for a total of 7 min. Under these conditions, folic acid eluted at approximately 7.6 min. The ESI was operated in the negative ion mode using selected ion monitoring (at *m/z* 440.2 and 445.2 for ^{12}C-folic acid and $^{13}C_5$-folic acid, respectively). The spray voltage was adjusted to 4.5 kV and capillary temperature set to 200 °C. The sheath and auxiliary gases were set to 55 and 15% of their maximum flow rate, respectively.

Confirmation of Folic Acid by Tandem Mass Spectrometry. Since the background matrix varied among the materials analyzed, the presence of folic acid in the cereal samples was confirmed using tandem mass spectrometry (MS/MS). Samples were injected into the instrument and the molecular anion, of folic acid at *m/z* 440 [M-H]$^-$, was selected and then subjected to collision induced dissociation (CID) at 26% of the maximum energy of the end cap electrode (5 V) of the ion trap. Using an isolation mass width of 1.2 amu, first generation ions produced by the collisions were collected in the full scan mode and the spectrum compared to that of authentic material.

RESULTS

Stability of Folic Acid in Storage. Both folic acid and the labeled-folic acid were stored 4 °C in 0.1 N NaOH without any noticeable deterioration over two months based on routine mass spectrometry analysis.

The Effect of Heat Treatment on Losses of Folic Acid. A heat treatment step for an hour was required to free the analyte from the food matrix. The extraction efficiency was greatly reduced if the heat treatment was omitted. To determine the optimal duration of the heat treatment, samples of the extract were taken at several time points during the heat treatment and analyzed. There was no improvement in extraction efficiency beyond an hour of heating. Additionally, there was about a 10% loss of the analyte as a result of this step.

LC/MS Analysis. A typical LC−MS chromatogram for folic acid and $^{13}C_5$-folic acid in a cereal matrix is given in Figure 2. The internal standard and analyte coeluted in a single chromatographic peak, which ap-

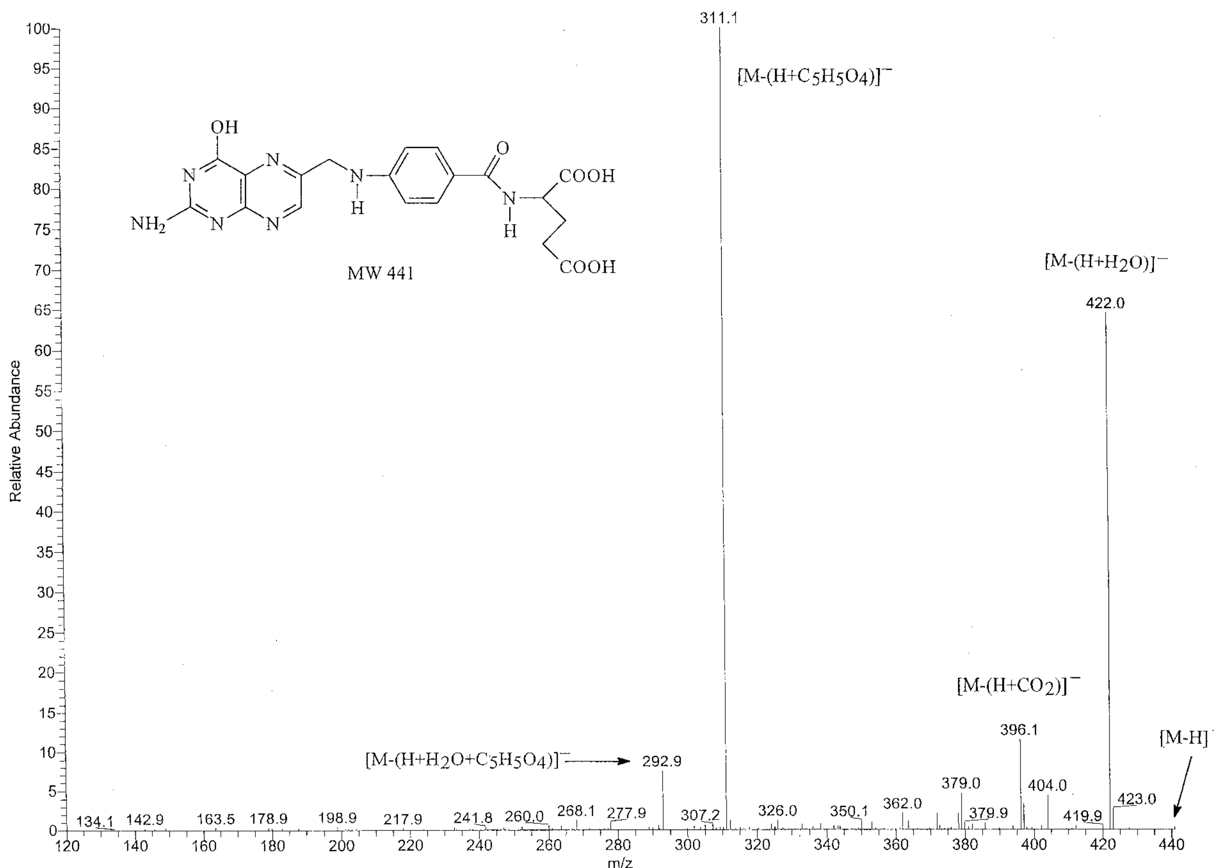

Figure 3. The collision induced mass spectrum of the molecular anion of folic acid as generated using electrospray ionization in the negative ion mode. The fragment ions represent logical fragmentation cleavages of the molecular anion that are given in the text.

Table 1. The Results of the LC−MS Determination of Folic Acid in a Group of Test Samples[a]

fortified food	μg/100 g	
	listed value	LC−MS
bran	400	420
corn	100	109
whole wheat	400	409
oat	100	107
SRM-1846	129	113

[a] Samples were extracted and analyzed by HLPC−MS using electrospray ionization in the negative ion mode. The values for folic acid were computed using a stable-isotope internal standard $^{13}C_5$-folic acid.

peared at about 7.6 min using the described chromatographic conditions. The upper panel of the figure presents the selected ion profile of the analyte (at *m*/*z* 440.2) as observed in the extract of a fortified cereal. The determination of folic acid using LC−MS was 109 μg per serving. This accorded well with a stated value of 100 μg per serving. The lower tracing depicts the chromatographic profile of the selected ion for the ^{13}C-labeled internal standard. A list of the determinations from a group of test samples is presented in Table 1. There was a high degree of concordance in the determinations from LC−MS analysis as compared with the stated claims for these products.

There was a small degree of isotopic overlap between the analyte and the internal standard. The isotopic contribution of folic acid to the ion current channel of the ^{13}C-labeled folic acid (at *m*/*z* 445.2) was small, representing approximately 1.6% of the total ion current abundance of the molecular anion (at *m*/*z* 440.2). For (unknown reasons) the reverse contribution of labeled-folic acid to the ion current channel of folic acid (at *m*/*z* 440.2) was of similar magnitude. The small isotopic contribution of the label to the ion channel of the analyte had no significant impact on the quantification of folic acid in the fortified foods that were tested. However, if samples contained low concentrations of folic acid then a calculation adjustment may be required to compensate for the isotopic contribution.

Precision. The precision of method was tested using SRM 1846 obtained from NIST. Currently, SRM 1846 is the only available material that has been given a reference value for folic acid. The mean value (for n = 4 determinations) as assessed using the LC−MS method was found to be 1.13 μg/g with a coefficient of variance (c.v.) of 5.6%. This value represented 88% of the total concentration of folates as given on the certificate (1.29 μg/g). SRM 1846 was certified for folates by independent laboratories using the microbiological (*L. casiei*) assay.

The higher values obtained by the microbiological method may be due to contributions from the naturally occurring folates present in the sample. The precision of the LC−MS method compared favorably with the values obtained by NIST from independent laboratories using the microbiological assay (c.v. approximately 22%).

Accuracy. The accuracy of the LC−MS analysis was determined using the SMOD of folic acid to a nonfortified wheat cereal. The range of the dilution curve covered a concentration ranging from 2.4 to 24 μg/g. Using least squares approximation, the best-fit regression to the empirical data was $y = 0.362x - 0.2441$; $R^2 = 0.991$. The accuracy was also determined by quantifying folic acid through a series of dilutions of the SRM

1846. The determined concentrations of folic acid through the dilution series corresponded to a linear function $y = 95x - 80$ with an R^2 of 0.997.

Confirmation of Folic Acid with Tandem Mass Spectrometry Analysis. The presence of folic acid in cereal products was confirmed based on the mass spectral analysis of the unique fragmentation pattern obtained using collision-induced dissociation of the molecular anion, [M-H, at *m/z* 440.2]$^-$ in the mass spectrometer. The molecular anion under these conditions exhibits significant fragmentation losses. As can be observed in the CID mass spectrum (Figure 3), the fragment ion at *m/z* 422 results from a loss of 18 mass units from the molecular anion at *m/z* 440, probably due to a loss of water. The ion at *m/z* 396 represents a loss of 44 amu and may be due to a loss of CO_2. The ions that appear at *m/z* 311 and 293 appear to be a loss of $C_5H_5O_4$ and ($C_5H_5O_4 + H_2O$), respectively. The rationalization of the fragment ions is consistent with structure of the folic acid accords with the most probable chemical cleavages of the anion undergoing collisions. The advantage of the tandem mass spectral analysis allows for confirmation of folic acid across a diverse selection of sample matrixes.

Determination of Folic Acid in Test Samples. The method was applied to the determination of folic acid in corn, wheat, bran, and milled-oat dry breakfast cereals as well as in SRM 1846. The results of the analysis are listed in Table 1 for these products. There was a high agreement between the amount of folic acid determined in the samples as compared to the stated values on the nutrition panel of the food label. One corn-based product claiming to contain 25% the daily folic acid requirement (approximately 100 μg per serving) was found to contain 109 μg per serving with a standard deviation of $\pm$ 6.6 μg for $n = 3$ determinations. The analysis of the whole grain wheat cereal was within 96% of the stated value on the food label based on LC−MS determinations. Other determinations are listed in Table 1.

DISCUSSION

The government mandate that required manufacturers to fortify grain-product foods with folic acid prompted the development of a specific and quantitative assay for folic acid in fortified foods for the purpose of acquiring accurate nutritional data for public health investigations. A stable-isotope dilution method based on mass spectrometry analysis was developed to determine the concentration of folic acid in fortified foods and a tandem mass spectrometry procedure was used to confirm the identity of the analyte. The accuracy of the method was evaluated using the standard method of addition of folic acid to a wheat-based cereal and tested against a Standard Reference Material that contained a referenced amount of folic acid. The accuracy of the method was shown to be linear over a concentration range, which encompassed the concentration of folic acid set forth in the mandate (140 μg of folic acid/100 g of food). A tandem mass spectrometry procedure was used to confirm the identity of folic acid in foods based on the unique fragmentation pattern of the collision-induced dissociation of the molecular anion formed in the electrospray. This method was applied to the determination of folic acid in several commercial cereal products that contained corn, wheat, and oat substrates. The values for the concentration of folic acid as determined by the LC−MS method were found to accord with the manufacturer's claims. This technique is advanced for the purpose of establishing a definitive procedure based on stable-isotope dilution mass spectrometry for the identification, confirmation, and quantification of folic acid in fortified foods. The extraction and purification procedures are relatively rapid and may be adapted to high through-put analyses. In addition, since mass spectrometry can supply unambiguous identification of folic acid, it may be used in conjunction with other assays to confirm the presence of the analyte.

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LITERATURE CITED

(1) U.S. Department of Health and Human Services, Food and Drug Administration. Federal Register, 5th March 1996 Part 3: 21 CFR parts 101, 136, 137, 138, 172; pp 8750−8807.

(2) Koehler, K. M.; Pareo-Tubbeh, S. L.; Romero, L. J.; Baumgartner, R. N.; Garry, P. J. Folate nutrition and older adults: challenges and opportunities. *A. Am. Diet. Assoc.* **1997**, *97*(2) 167−73.

(3) Tucker, K. L.; Mahnken, B.; Wilson, P. W.; Jacques, P.; Selhub, J. Folic acid fortification of the food supply. Potential benefits and risks for the elderly population *JAMA* **1996**, *276*(23), 1879−85.

(4) Wright, A., J.; Phillips, D. R. The threshold growth response of Lactobacillus casei to 5-methyl-tetrahydrofolic acid: implications for folate assays. *Br. J. Nutr.* **1985**, *53*(3), 569−73.

(5) Pfeiffer, C. M.; Rogers, L. M.; Gregory, J. F. Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reverse-phase liquid chromatography. *J. Agric. Food Chem.* **1997**, *45*, 407−413.

(6) Konings, E. J. M. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. *J. AOAC Intl.* **1999**, *82*, 119−127.

(7) Seyoum, E.; Selhub, J. Combined affinity and ion pair column chromatographies for analysis of food folate. *J. Nutr. Biochem.* **1993**, *4*, 448−494.

(8) Cheruppolil, R. S-K.; Kolhouse, J. F. Molar quantitation of folates by gas chromatography−mass spectrometry. *Methods Enzymol.* **1997**, *261*, 26−34.

(9) Garbis, S. D.; van Breemen R. B. The analysis of folic acid in human plasma using LC-MS. 47th Annual Conference on Mass Spectrometry, 2000.

(10) Holcapek, M.; Jandera, P. Interpretation of electrospray and atmospheric pressure chemical ionization mass spectra of 10-fromyl-7,8-dihydrofolic acid and 5-fromyl-5,6,7,8-terahydropteroic acid. *Rapid Comm. Mass. Spec.* **1999**, *13*, 1423−26.

(11) Stokes, P.; Webb, K. Analysis of some folate monoglutamates by high-performance liquid chromatography−mass spectrometry. I *J. Chromatogr. A.* **1999**, *864*, 59−67.

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